International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61L 27/00, 15/28	A1	(11) International Publication Number: WO 96/33750 (43) International Publication Date: 31 October 1996 (31.10.96)

(21) International Application Number: PCT/EP96/01734

(22) International Filing Date: 25 April 1996 (25.04.96)

(30) Priority Data:
PD95A000083 27 April 1995 (27.04.95) IT

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(81) Designated States: AL, AM, AU, AZ, BB, BG, BR, BY, CA, CN, CZ, EE, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ARTIFICIAL SKIN CONTAINING AS SUPPORT BIOCOMPATIBLE MATERIALS BASED ON HYALURONIC ACID DERIVATIVES

(57) Abstract

Artificial human skin comprising: a) microperforated membrane based on a hyaluronic acid derivative, on which keratinocytes have been seeded and cultured, b) an underlying non-woven tissue based on a hyaluronic acid derivative wherein fibroblasts have been seeded and left to proliferate. The artificial human skin according to the present invention can be therefore advantageously used in medicine, in surgery, in diagnostics and as a vehicle for preparing a controlled release medicament. Lastly, this artificial human skin can be frozen to -80 °C or placed in liquid nitrogen and stored, so that a tissue bank can be created.

ARTIFICIAL SKIN CONTAINING AS SUPPORT BIOCOMPATIBLE MATERIALS BASED ON HYALURONIC ACID DERIVATIVES

FIELD OF THE INVENTION

The present invention relates to:

- an artificial human skin constituted by a completely differentiated epidermis and dermal appendage, and containing as the support two biocompatible materials based on hyaluronic acid (HA) derivatives
- the processes for preparing this artificial human skin,
- the use of this artificial skin in medicine and in surgery, as a diagnostic device, and as vehicling agent for preparing controlled release medicament.

BACKGROUND OF THE INVENTION

Skin loss due to trauma or disease is usually treated by the autograft technique, that is, by substituting the missing skin with pieces taken from donor areas of the same patient. An important step forward in the treatment of such lesions by reconstructive surgery is represented by *in vitro* cultures of keratinocytes (kc) (J. Rheinwald and H. Green, Cell, 6:331, 1975), whereby said cultures are allowed to expand *in vitro*, and membranes of epidermal cells are obtained which are potentially useful to cover skin wounds. This technique has been widely used in clinical practice, mainly for burn patients (G. G. Gallico et al., M. Engl.J. Med., 311-448, 1984), but problems arose right from the start, such as the difficulty for such grafts to take, the fragility of the epithelial sheets and consequent difficulty for the surgeon to handle them.

substitutes which do not have these drawbacks, and satisfy the following requirements:

1) their surfaces must allow for adhesion and cell growth;

2) neither the polymers themselves, nor their degradation products should cause inflammation or toxic phenomena when implanted *in vivo*;

3) the product should be perfectly reproducible in its three dimensions;

4) its ideal porosity is 50%, which gives a large surface area for cell-polymer interactions, sufficient volume for the deposit of extracellular matrix and only slight, or no, migrational impediments during *in vitro* culture.

5) the supporting polymer should be absorbed once the regenerated tissue no longer requires it. Indeed, foreign bodies *in vivo* represent a high risk of infection and/or inflammation.

Indeed, the products already on the market or being developed present certain drawbacks: their degradation is uncontrollable and interferes with the wound healing process, thus favouring inflammation. Moreover, these substitutes require the epithelial cells to be thickly seeded on the support and left to proliferate for a long time.

Some examples of the products known to date and generally recommended for use in treatment of severe burns are:

1) Dermagraft®, developed by ATS (California), wherein heterologous human fibroblasts are cultivated on a spongy, resorbable material constituted by polylactic, polyglycol or polygalactoside acid. Autologous kc are seeded onto these materials;

2) GraftSkin®, produced by Organogenesis Inc. (Boston, USA), wher in heterologous human fibroblasts are cultivated on a collagen based

until it disappears in the completely differentiated kc (Carter et al., J. Cell Biol. 113207, 1991). Moreover, the role of CD44 in HA's degradation mechanism is well documented (Culty et al. J. Cell. Biol. 111:2765, 1990; Underhill, Dev. Biol. 155:324, 1993).

The purpose of the present invention is an artificial human skin simulating both the epidermal and dermal layer of the natural one, wherein both fibroblasts and keratinocytes (kc) are present, both cell types actively proliferating and separated, at the interface, by a protein extracellular matrix, having the characteristic of a dermoepidermal junction.

SUMMARY OF THE INVENTION

The Applicant has therefore unexpectedly found the artificial human skin subject of the present invention which comprises:

a) a microperforated membrane based on a hyaluronic acid derivative, on which keratinocytes have been seeded and cultured,

b) an underlying non-woven tissue based on a hyaluronic acid derivative wherein fibroblasts have been seeded and left to proliferate.

The artificial skin, subject of the present invention generally further comprises an extracellular protein matrix containing proteins of the dermo-epidermal junction, placed at the interface between keratinocytes and fibroblasts.

Thanks to its biodegradability, which can also be a result of the receptor mechanism mentioned previously, the artificial human skin is spontaneously absorbed within a set time, leaving just the newly-formed tissue at the lesion site.

Therefore the artificial human skin according to the present invention

preparing said human artificial skin.

In particular the first process comprises the following steps:

i) seeding keratinocytes cultures at a density ranging from 1,000 to 100.000 cell/cm^2, on the microperforated membranes and expanding the Kc on said membrane. The conventional culture technique involving the use of Foetal Calf Serum (FCS) is followed until partial or complete confluence is achieved.

ii) cultivating the fibroblasts isolated from the dermis or from other districts by the usual techniques in DMEM containing 10% foetal calf serum, conditioning these fibroblasts to proliferate first by seeding them on plastic, and then seeding the fibroblasts in the non-woven tissue and continuing the culture until freezing, (in case the obtained artificial skin must be preserved) or until grafting,

iii) laying the epithelial layer formed on the microperforated membrane on the non-woven tissue colonized by fibroblasts, being careful that the outer edge of the microperforated membrane does not touch the bottom of the dish and that it extends slightly beyond the underlying non-woven tissue,

iv) optionally fixing together the epithelial cells layer on the microperforated membrane to the underlying non-woven tissue colonized by fibroblasts by means of biological adhesives selected from the group consisting of: collagen, fibrin, fibrin glue.

v) adding a sufficient volume of the same medium used in step (i), keeping the culture immersed so that the keratinocytes of the upper layer of the microperforated membrane are at the air-liquid interface, adding at the first and subsequent changes of medium, ascorbic acid at concentration equal to 1µg/ml, continuing the culture until

in the presence of a cryopreserving agent, when the skin must be pr served.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows Hematoxylin and Eosin staining of a 15-day-old composite dermal-epidermal culture. Kcs have migrated through the Laserskin[R] membrane to the non-woven tissue, which is embedded with fibroblast cells (x100).

Fig. 2 shows the immunohistochemistry of the composite culture probed with an antiinvolucrin antibody, the outer epithelial layer is undergoing keratinization (x 200).

Fig. 3 shows the immunohistochemistry of the composite culture probed with anti-KL4 (total keratin) antibody, a classic Kc marker (x 200).

Fig. 4a shows the immunohistochemistry of the composite culture showing the β4 integrin subunits expression, predominantly found in the basal Kc cell layer (x 100).

Fig. 4b shows the immunohistochemistry of the composite culture showing the β1 integrin subunit mainly expressed at the intercellular bridges joining the epithelial cells (x 100).

Fig. 5 shows the immunolocalization of basal Kcs expressing the CD44 receptor (x 200).

Fig. 6 shows the immunohistochemistry of the composite culture showing the basement-membrane-like zone, as can be seen from the extent of laminin deposition (x 200).

DETAILED DESCRIPTION OF THE INVENTION

The epithelial layer is reconstructed by seeding human kc on a microperforated membrane, prepared according to USP 5,326,356, which we incorporate by reference, while the human fibroblasts are

Indeed, a physiological covering for these kinds of lesions, albeit composed of heterologous cells, reduces the risk of infection or excessive loss of organic fluids. Moreover, the proliferating cells in the dermal bed secrete substances (still partly unknown) such as growth factors and cytokines, which accelerate the wound healing process.

Moreover the skin according to the present invention can be used for the treatment of diseases characterized by melanine deficiency such as vitiligo.

In this case the artificial human skin of the present invention contains a quota of melanocytes which can be grafted by means of the same skin to patients affected by vitiligo.

The artificial human skin can in particular also be applied in genetic therapy, wherein the cells of a patient (kc, fibroblasts or both) are genetically modified, for example to correct congenital malformations and/or metabolic defects.

When the human artificial skin according to the present invention is used as vehicling agent for preparing controlled release medicaments containing proteins, these are preferably growth factors, such as FGF (Fibroblast Growth Factors), TGF β(Transforming Growth Factor β), KGF (Keratinocyte Growth Factor), NGF (Nerve Growth Factor), proteins involves in the clothing cascade such as Coagulation Factor VIII and others. Moreover, proteins of the extracellular matrix, such as Fibronectin, Laminin, Collagen can be adsorbed to the fibres of the non-woven tissue, to improve the cell attachment process.

In the first process according to the present invention , in step (i) the seeding density of keratinocytes cells is preferably comprised

examined the expression of CD44, and found that the protein is localized in the innermost basal layers of the composite and, above all, in the epithelial cells in direct contact with Hyaff 11 (benzyl ester of HA), both in the form of non-woven tissue and in the form of microperforated membranes. This suggests that, besides acting as a support for the cells, the biomaterial also interacts with the cells which adhere to it.

Lastly, it is possible to observe the production of specific proteins of the dermoepidermal junction (laminin, collagen, types III, IV and VII), while on the "dermal" side there is abundant production of the extracellular matrix, shown by specific staining and immunohistological methods (see example 1).

For purely indicative purposes, and without being limited by the same, we report here some examples characterising the new-formed tissue.

Example 1

A piece of non-woven tissue (1.5 x 1.5 cm) constituted by Hyaff 11 (benzyl ester of HA) is laid on the bottom of a culture dish and fixed in place at the four corners with fibrin glue. Primary human fibroblasts are seeded on the non-woven tissue (0.1×10^6 cells in 0.2 ml of medium) slowly soaking the non-woven tissue. The dish is covered and left to stand for about 30 minutes under a sterile hood, after which the volume is brought to 2 ml with DMEM containing 10% FCS and the dish is incubated at 37°C in an atmosphere of 5% CO_2.

Human kc between the II and IV culture passages are seeded on a Laserskin^R membrane measuring 5 x 5 cm^2 (Andreassi et al. Wounds 3:116, 1991). The kc can be cultivated in CEC medium (Rheiwald and Green, Cell, 6:331, 1975) in the presence or in the absence of a

cut in half with a scalpel, being careful to maintain its structure. One half is treated by traditional histological techniques (hematoxylin-eosin staining), while the other half is immersed in OCT (medium for the cryopreservation of tissues, Milestones, USA), frozen in liquid nitrogen and then stored at -80°C. The latter is cut into 6 µm thick slices with a cryostat. The slices are used for immunohistochemical investigation, using antibodies against the markers of the differentiation status of the kc, of the dermal-epidermal junction and of the extracellular matrix.

Results of histological hematoxylin-eosin staining

Figure 1 (x100) shows a well stratified epithelium over a microperforated Laserskin® membrane. The upper and outer layer is undergoing keratinization. The kc appear to be well distributed on the underside of the membrane, and to have colonized the underlying nonwoven tissue to a considerable extent. The epithelial layer is very compact with a variable thickness which is never less than 10-15 cells. The dividing line between the layer of epithelial cells and underlying fibroblasts can be seen clearly. The cross-sections of fibres from the non-woven tissue are also clearly visible, as is the Laserskin® microperforated membrane.

Immunohistochemical characterization

Studies on the tissue markers were conducted with the following antibodies:

1) anti-keratin antibody KL4 (Dermatology Department, *Immacolata*, Rome)

2) anti-involucrin polyclonal antibody (Dermatology Department, *Immacolata*, Rome)

d) The kc in direct contact with the fibers of the non-woven tissue, like the cells in contact with the top and underside of the Laserskin®, show a marked immunopositivity to CD44 (Fig. 5, x200). It is thought that this represents a singular feature of the composite culture described here: indeed, CD44 can mediate cell adhesion to the material (constituted by modified hyaluronic acid), and likewise its degradation.

e) In the basement membrane, and therefore also the dermoepidermal junction, a typical extracellular matrix can be observed, the components of which are mainly produced by the basal kc resident in the area. Characteristic markers are therefore laminin and collagen types IV and VII. Confirmation of these observations is given by the fact that the three components are expressed in the interposition between the basal layer of the kc embedded in the non-woven tissue and the underlying fibroblasts. In particular, the extent of the response to laminin (Fig. 6, x200) is focalized on that which can be considered an "artificial basement membrane".

f) Lastly, on the side of the dermis, a strong immunopositivity to fibronectin can be found. Fibronectin is a protein of the matrix produced by the fibroblasts which are obviously abundant in the dermis. The lack of response in the epidermal layer again indicated that the formation of a well-structured dermal tissue with the typical natural skin structure has been artificially induced.

Example 2

In order to demonstrate the possibility of cryopreserving the artificial skin thus produced, the dermal-epidermal cultures are frozen in the presence of a cryopreserving agent such as

CLAIMS

1. Artificial human skin comprising:
a) a microperforated membrane based on a hyaluronic acid derivative, on which keratinocytes have been seeded and cultured,
b) an underlying non-woven tissue based on a hyaluronic acid derivative wherein fibroblasts have been seeded and left to proliferate.

2. The artificial human skin according to claim 1 further comprising an extracellular protein matrix, containing proteins of the dermoepidermal juntion placed at the interface between fibroblasts and keratinocytes cells.

3. The artificial human skin according to one of the claims 1 and 2 wherein said hyaluronic acid derivative used for preparing both the microperforated membrane and the non-woven tissue is an ester of hyaluronic acid.

4. The artificial human skin according to claim 3 wherein said hyaluronic acid ester has a percentage of esterification comprised between 75% and 100%.

5. The artificial human skin according to claims 3 and 4 wherein said ester is the hyaluronic benzyl ester.

6. The artificial human skin according to one of the claims 1-5, for use in medicine and in surgery.

7. The artificial human skin according to claim 6 for use on deep, II and III-degree burns, for use on various kinds of ulcer selected from the group consisting of: diabetic, venous, bedsores, for use in plastic surgery and for use in diseases characterized by melanine deficiency.

of claims 1-5 comprising the following steps :

i) seeding keratinocytes cultures at a density ranging from 1.000 to 100.000 cell/cm^2, on a microperforated membrane based on a hyaluronic acid derivative and expanding said Kc on said membrane according to conventional techniques involving the use of foetal calf serum, until achieving partial or complete confluence,

ii) cultivating the fibroblasts isolated from the dermis or from other districts by the usual techniques in DMEM containing 10% foetal calf serum, conditioning these fibroblasts to proliferate by seeding on plastic, seeding the fibroblasts in the non-woven tissue and continuing the culture until freezing, (in case the obtained artificial skin must be preserved) or until grafting.

iii) laying the epithelial layer already formed on the non-woven tissue colonized by fibroblasts, being careful that the outer edge of the microperforated membrane does not touch the bottom of the dish and that it extends slightly beyond the underlying non-woven tissue,

iv) optionally fixing together the epithelial cells layer on the microperforated membrane to the underlying non-woven tissue colonized by fibroblasts by means of biological adhyesives selected from the group consisting of: collagen, fibrin, fibrin glue.

v) adding a sufficient volume of the same medium used in step (i), keeping the culture immersed so that the keratinocytes of the upper layer of the microperforated membrane are on the air-liquid interface, adding at the first and subsequent changes of medium, ascorbic acid at concentration equal to 1µg/ml, continuing the culture unitil graft or freezing.

15. The process according to claim 14 wherein in step (i) the seeding

FIG. 1

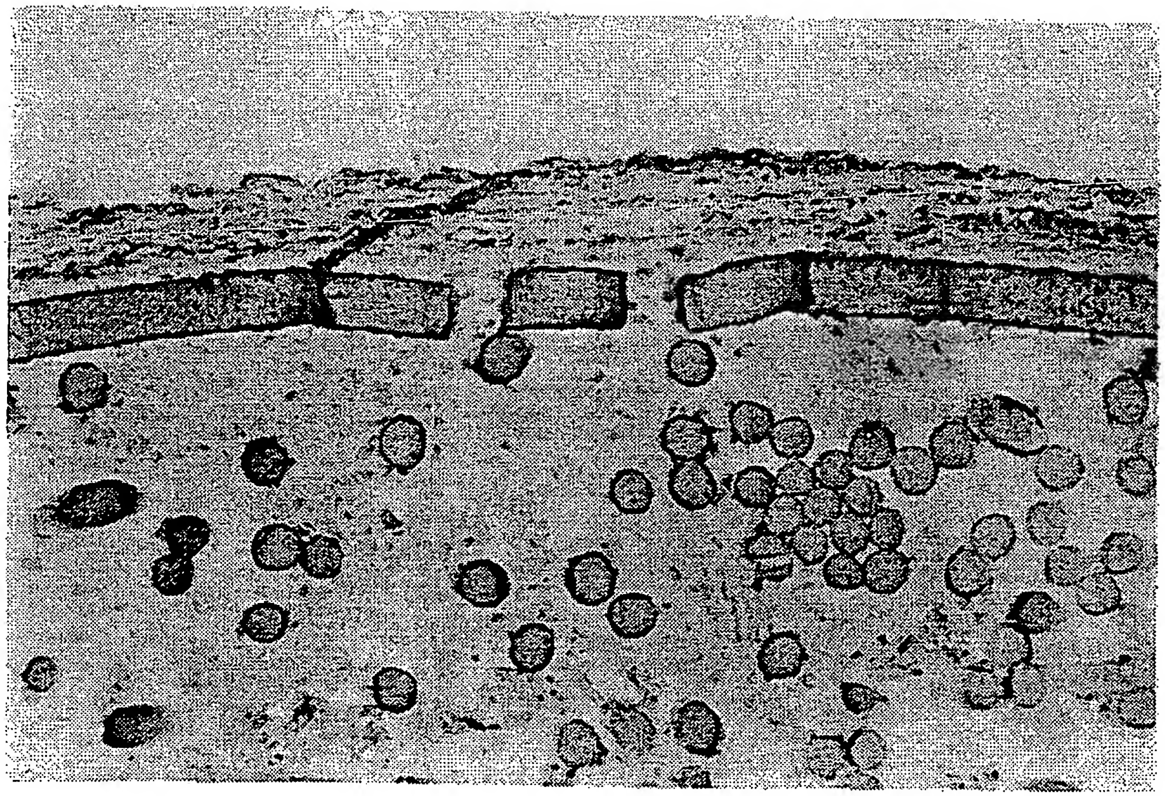

FIG. 2

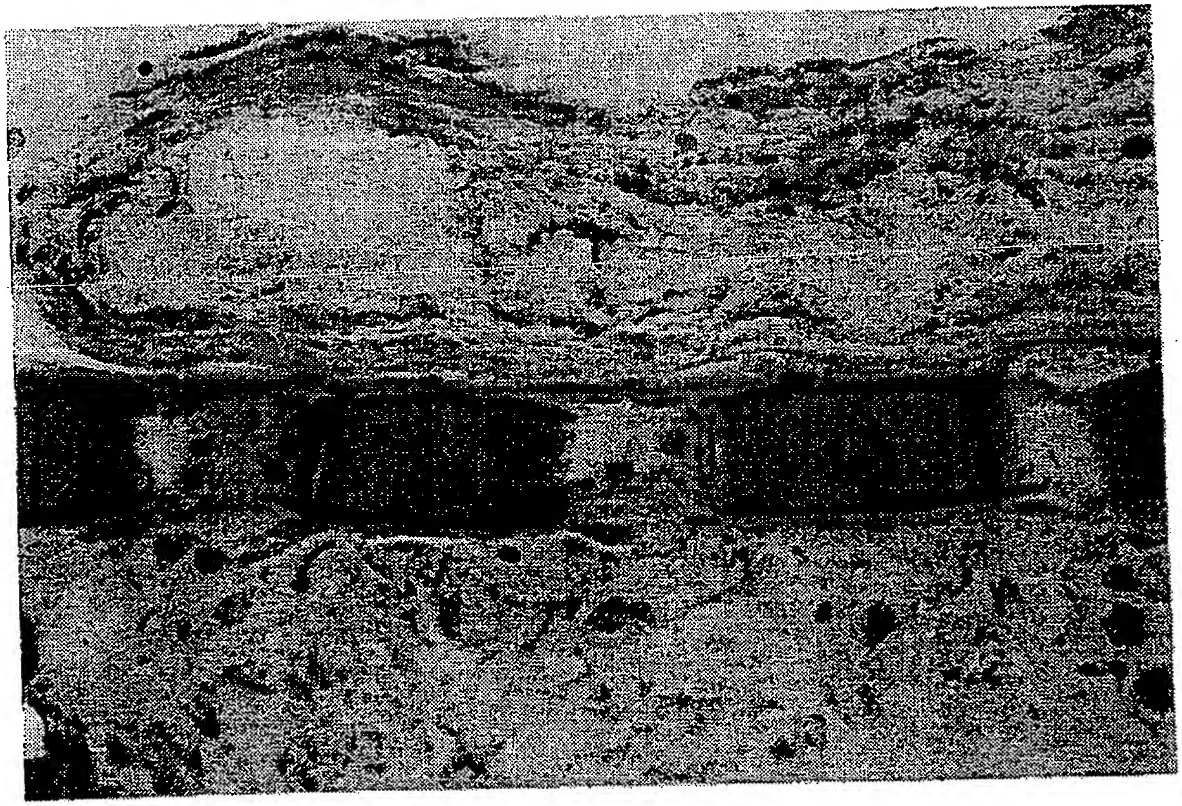

FIG. 3

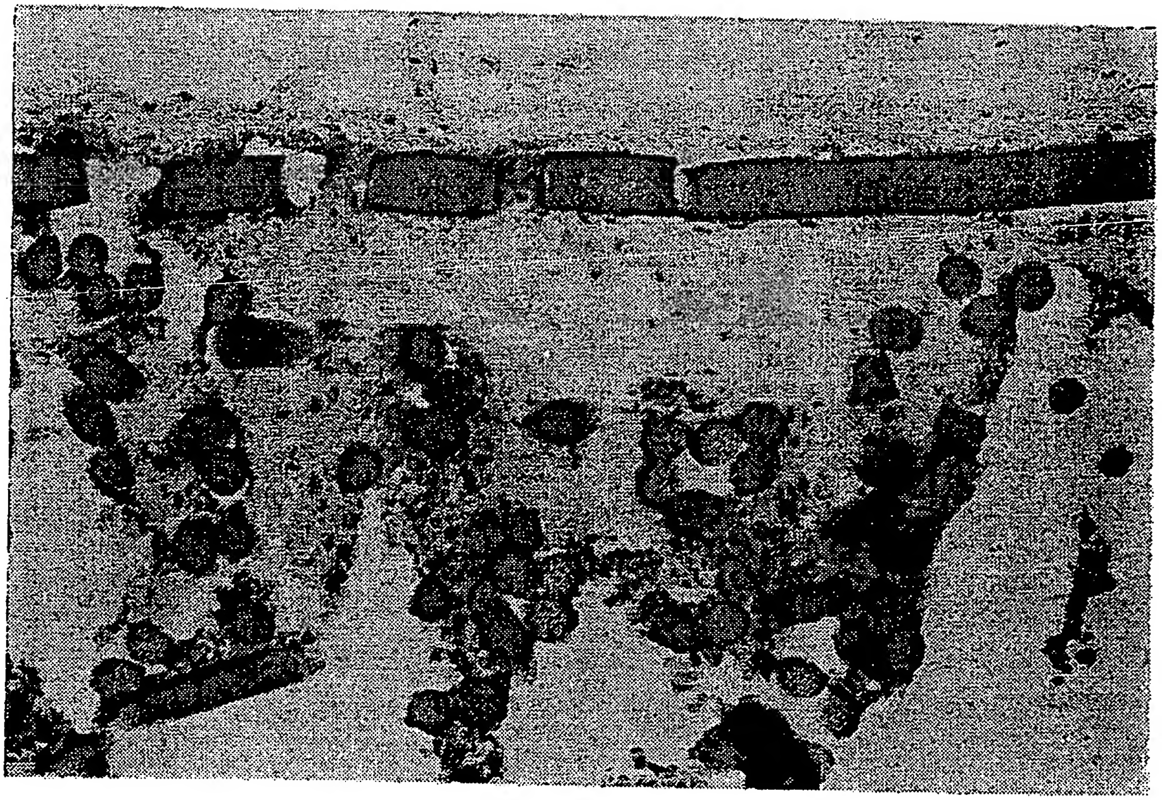

FIG. 4A

FIG. 4B

FIG. 5

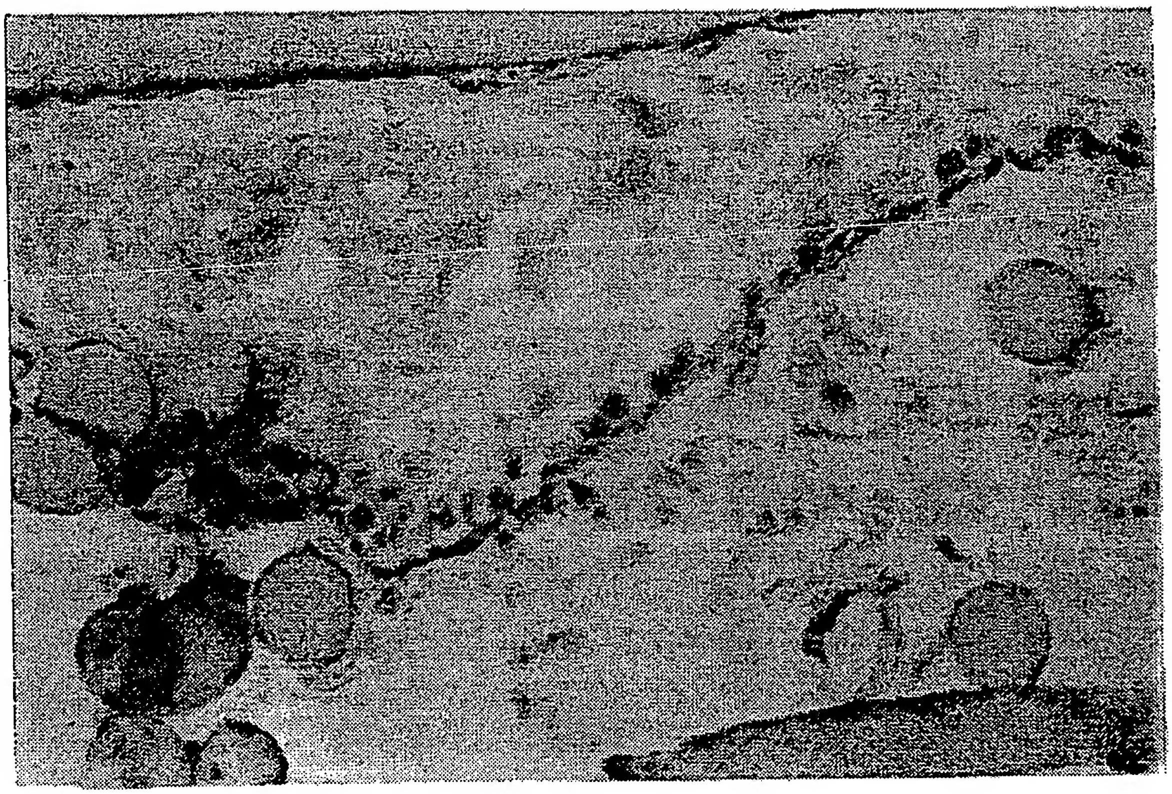

FIG. 6

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61L27/00 A61L15/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 462 426 (FIDIA) 27 December 1991 cited in the application see claims 7,11-13,19,20 ---	1-18
A	EP,A,0 265 116 (FIDIA) 27 April 1988 see claim 23 ---	1,6,7
A	WO,A,91 16010 (EISENBERG M.) 31 October 1991 -----	

☐ Further documents are listed in the continuation of box C.

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1

Date of the actual completion of the international search	Date of mailing of the international search report
11 September 1996	18.09.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Peltre, C

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61L27/00 1L15/28

According to International Patent Classification (IPC) or to both national classification and IPC

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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Peltre, C